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Corneal endothelial regeneration and tissue engineering

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ABSTRACT

Human corneal endothelial cells (HCECs) have a limited proliferative capacity. Descemet stripping with automated endothelial keratoplasty (DSAEK) has become the preferred method for the treatment of corneal endothelial deficiency, but it requires a donor cornea. To overcome the shortage of donor corneas, transplantation of cultured HCEC sheets has been attempted in experimental studies. This review summarizes current knowledge about the mechanisms of corneal endothelial wound healing and about tissue engineering for the corneal endothelium. We also discuss recent work on tissue engineering for DSAEK grafts using cultured HCECs and HCEC precursor cell isolation method (the sphere-forming assay). DSAEK grafts (HCEC sheets) were constructed by seeding cultured HCECs on human amniotic membrane, thin human corneal stroma, and collagen sheets. The pump function of the HCEC sheets thus obtained was approximately 75%–95% of that for human donor corneas. HCEC sheets were transplanted onto rabbit corneas after DSAEK. While the untransplanted control group displayed severe stromal edema, the transplanted group had clear corneas throughout the observation period. The sphere-forming assay using donor human corneal endothelium or cultured HCECs can achieved mass production of human corneal endothelial precursors. These findings indicate that cultured HCECs transplanted after DSAEK can perform effective corneal dehydration in vivo and suggest the feasibility of employing the transplantation of cultured HCECs to treat endothelial dysfunction. Additionally, corneal endothelial precursors may be an effective strategy for corneal endothelial regeneration.

Contents

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1. Introduction

1.1. Structure and function of corneal endothelium

Corneal endothelial cells (CECs) are believed to arise from the neural crest (Johnston et al., 1979; Bahn et al., 1984) and form a monolayer of hexagonal cells that acts as a barrier between the corneal stroma and the aqueous humor in the anterior chamber. Transparency of the cornea is maintained by regulating stromal hydration through the barrier and pump functions of the corneal endothelium. Human CECs (HCECs) normally display limited proliferative capacity in vivo (Wilson et al., 1993, 1995; Egan et al., 1998; Senoo and Joyce, 2000; Senoo et al., 2000), because they are arrested in Gl phase (Joyce et al., 1996a,b). Therefore, the number of HCECs gradually decreases with age (Murphy et al., 1984; Bourne et al., 1997; Hollingsworth et al., 2001) and declines dramatically after endothelial damage due to trauma, phacoemulsification, or acute angle-closure glaucoma. Corneal endothelial damage has been directly implicated in causing bullous keratopathy because of the relative lack of endothelial cell proliferative capacity. Penetrating keratoplasty (PKP) has long been performed as the initial treatment for CEC dysfunction. In fact, more than half of the patients who undergo full-thickness corneal transplantation have impaired visual acuity that is entirely due to corneal endothelial problems and their corneal epithelium is normal (Mannis and Krachmer, 1981; Rapuano et al., 1990; Cosar et al., 2002). Corneal transplantation requires a fresh human cornea, but there is a worldwide shortage of donors.

1.2. History of corneal endothelial tissue engineering

To overcome this donor cornea shortage, transplantation of cultured HCEC sheets has been tested in experimental studies as a substitute for full-thickness corneal transplantation. Cultured HCECs derived from adult human donor corneas have been transplanted onto denuded Descemet's membrane (Insler and Lopez, 1986, 1991a,b; Engelmann and Friedl, 1989; Engelmann et al., 1999; Aboalchamat et al., 1999; Bohnke et al., 1999; Chen et al., 2001; Amano, 2002, 2003; Mimura et al., 2004a), collagen matrix (Mimura et al., 2004b), amniotic membrane (Ishino et al., 2004), human corneal stromal discs (Honda et al., 2009; Choi et al., 2010), gelatin hydrogel discs (Lai et al., 2007; Watanabe et al., 2011), and chitosan-based membrane (Liang et al., 2011) ex vivo. Although culture of HCECs from donor corneas yields cells with HCEC-like morphology and function, the cultured cells become increasingly heterogeneous with an increase of donor age or a greater number of passages (Miyata et al., 2001; Zhu and Joyce, 2004; Joyce and Zhu, 2004). In addition, the density of HCECs, which is a pivotal factor in maintaining long-term corneal transparency, decreases after transplantation of HCEC sheets as well as after conventional PKP (Mimura et al., 2004b). Thus, a high cell density and normal hexagonal cells with adequate endothelial function are crucial requirements for obtaining cultured HCEC sheets that are comparable with or better than donor corneas.

1.3. Use of cultured HCECs for Descemet stripping with automated endothelial keratoplasty

Over the last few years, Descemet stripping with automated endothelial keratoplasty (DSAEK) has become a standard procedure for corneal transplantation in patients with endothelial dysfunction (Gorovoy, 2006; Koenig and Covert, 2007; Price et al., 2008; Terry et al., 2008). DSAEK achieves better postoperative visual function and reduces the risks associated with penetrating keratoplasty, such as severe astigmatism and expulsive hemorrhage. Furthermore, Descemet membrane endothelial keratoplasty (DMEK) and Descemet membrane automated endothelial keratoplasty (DMAEK) are the next steps for endothelial keratoplasty (Terry, 2012). This procedure can provide faster visual rehabilitation, minimize postoperative ocular surface complications, and preserve endothelial cell density compared DSAEK (Dapena et al., 2011; Guerra et al., 2011; Laaser et al., 2012; Parker et al., 2012; Naveiras et al., 2012; Krabcova et al., 2012; Terry, 2012). However, DSAEK, DMEK, or DMAEK require a donor cornea, so the worldwide shortage of donor corneas limits its application. If cultured HCECs could be used for corneal transplantation, many patients with corneal endothelial dysfunction could be treated by using cells from a single donor cornea. Therefore, he feasibility of DSAEK using cultured HCECs has been investigated (Mimura et al., 2004b; Honda et al., 2009). In this review, we discuss the mechanisms of corneal endothelial wound healing and provide an overview of tissue engineering for the corneal endothelium with cultured CECs, focusing on recent studies into the feasibility of transplanting cultured HCEC sheets and human corneal endothelial precursors. All studies described here were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Human donor corneas were handled according to the tenets of the Declaration of Helsinki of 1975 and its 1983 revision.

2. Healing of corneal endothelium

2.1. Growth capacity of HCECs

Healing of corneal endothelial wounds occurs predominantly by migration and enlargement of existing cells rather than by

proliferation to replace dead cells (Matsubara and Tanishima, 1983; Joyce et al., 1989; Joyce, 2003). This type of healing, as well as the fact that the corneal endothelial cell density decreases with age, strongly suggests that corneal endothelial cells do not normally proliferate in vivo. Studies performed at Joyce's laboratory (Senoo and Joyce, 2000; Senoo et al., 2000; Chen et al., 2001; Zhu and Joyce, 2004; Joyce and Zhu, 2004) and other laboratories (Baum et al., 1979; Engelmann et al., 1988) have indicated that human corneal endothelial cells (HCEC) retain the ability to proliferate, but are strongly inhibited from doing so in vivo. Work done at Joyce's laboratory has also demonstrated an intrinsic age-related difference in the growth capacity of HCECs using both an ex vivo corneal wound model (Senoo and Joyce, 2000) and cultured HCEC (Chen et al., 2001; Zhu and Joyce, 2004; Joyce and Zhu, 2004; Konomi et al., 2005).

2.2. Comparison of proliferative capacity in central and peripheral areas of endothelium

Several groups have studied HCEC replication and the density of these cells in the peripheral and central areas of the cornea. Tissue culture studies performed by Bednarz et al. (1998) have indicated that HCEC from the corneal periphery are able to replicate, while cells from the corneal center exhibit little or no mitotic activity.

Schimmelpfennig et al. and Amann et al. reported that the density of HCEC is higher at the periphery than the center of the cornea, while the density decreases in both areas as the donor age becomes older (Schimmelpfennig, 1984; Amann et al., 2003). It is also well known that the density of HCECs decreases gradually after birth. Thus, it is possible that HCECs from the central cornea become more stressed than peripheral cells with aging, leading to loss of proliferative capacity and a more senescent phenotype.

2.3. Evaluation of replication competence using minichromosome maintenance protein-2

Konomi and Joyce compared regional differences of proliferative capacity after culturing HCEC isolated from the central and peripheral corneas of younger (<30 years) and older (>50 years) donors (Konomi et al., 2005). The relative proliferative capacity was determined by comparing the population doubling time and by immunostaining for minichromosome maintenance protein-2 (MCM2) as a marker of replication competence (Todorov et al., 1995, 1998; Wharton et al., 2001). These studies showed no statistical difference of the population doubling time or replication competence between central and peripheral HCECs within either age group, although central cells (particularly from older donors) exhibited a somewhat longer population doubling time. Their results indicated that cultured HCEC from both the central and peripheral cornea retained proliferative capacity.

Mimura and Joyce also previously compared replication competence and senescence between HCECs from the central and peripheral areas of the cornea and between cells from younger (<30 years) and older donors (>50 years) (Mimura and Joyce, 2006). After a 2-mm wound was created in human corneal endothelium running from the periphery to the center, cells repopulated the wound over time in both age groups. In older corneas, however, significantly fewer HCEC migrated into the wound bed at the central region than at the periphery. Also, the density of cells in the central part of the wound was always lower in corneas from older donors than in those from younger donors (Fig. 1). In both age groups, the mean percentage of MCM2-positive cells increased over time until the wound was healed. In both age groups, more MCM2-positive cells were detected in the peripheral cornea than in the central cornea, but the percentage of MCM2-positive cells in the central or peripheral areas was significantly lower in older corneas than in the corresponding regions of younger corneas (Fig. 1). Senescence-associated β-galactosidase (SA-β-Gal) is widely used as a biomarker of replicative senescence (Dimri et al., 1995). HCECs from the corneas of younger donors showed little or no SA-β-Gal activity in either the central or peripheral areas. In contrast, SA-β-Gal activity was easily detectable in HCECs from older donors and a significantly higher percentage of central cells showed strong SA-β-Gal activity compared with cells from the periphery. These results suggested that peripheral HCECs retain more replication competence than central HCECs regardless of donor age. In addition, significantly fewer central HCECs retain the ability to replicate in corneas from older donors compared with the central cells of corneas from younger donors, and HCECs (particularly those in the central cornea) undergo senescence-like changes with advancing donor age. Taken together, these findings indicate that greater loss of cells from the central corneal endothelium leads to gradual rearrangement and the centripetal movement of cells from the denser periphery toward the center. Such movement recapitulates the process of embryonic development of the cornea, in which neural crest cells migrate centripetally and differentiate into mature corneal endothelium (Bard et al., 1975; Johnston et al., 1979; Meier, 1982).

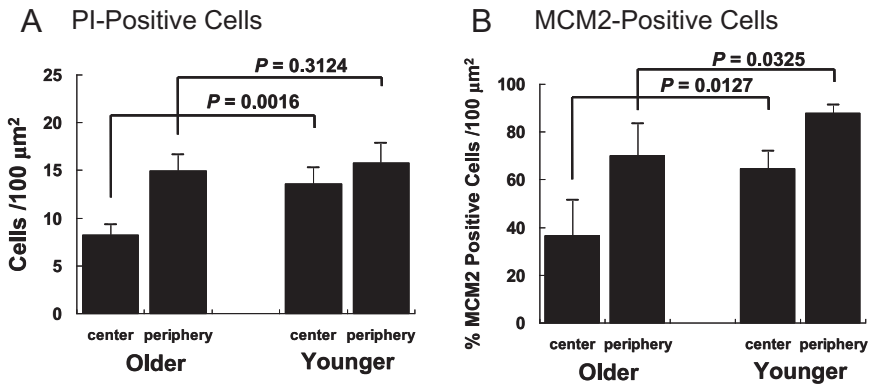

Fig. 1. Comparison of replication in a corneal endothelial wound model between younger and older corneas (modified from [Mimura and Joyce, 2006] with permission). A 2-mm-wide wound was made in the corneal endothelium, followed by incubation in culture medium for 72 h. The cell density was determined by measuring the fluorescence intensity of propidium iodide (PI) for DNA staining to enable visualization of cells. The density of PI-positive HCECs (A) and cells positive for minichromosome maintenance (MCM)-2 was compared between corneas from younger ($n = 4$, aged 30 years or less) and older ($n = 4$, aged 50 years or more) donors. Data are presented as the mean ± SD. The unpaired Student's *t*-test was used to compare mean values.

3. Culture of HCECs

3.1. Growth factors

Several groups have established HCEC culture techniques (Engelmann and Friedl, 1989; Yue et al., 1989; Chen et al., 2001; Miyata et al., 2001; Ishino et al., 2004; Li et al., 2007, Table 1). Various growth factors have been reported to influence the proliferation of cultured cells obtained from human corneal endothelium, including fibroblast growth factor (Engelmann et al., 1988, 1989, 1995; Yue et al., 1989; Samples et al., 1991; Chen et al., 2001), epidermal growth factor (Yue et al., 1989; Samples et al., 1991; Schultz et al., 1992; Chen et al., 2001), nerve growth factor (Chen et al., 2001), and endothelial cell growth supplement (Yue et al., 1989; Blake et al., 1997).

Recently, Shima et al. developed a simple culture method for HCECs using L-ascorbic acid 2-phosphate (Asc-2P) (Shima et al., 2011). They studied the influence of various mitogens, extracellular matrices (ECMs), and Asc-2P on the growth of cultured HCECs. Incubation of cells on atelocollagen with Asc-2P and bFGF promoted HCEC proliferation in both primary culture and subculture as efficiently as conventional culture with ECM derived from bovine corneal endothelial cells. During multiple passages, cells cultured without Asc-2P showed a decrease of growth and developed an irregular morphology, whereas cells cultured with Asc-2P maintained both their growth and their characteristic polygonal morphology. Asc-2P increased the proliferation and replicative lifespan of HCECs from donors over a wide age range. Moreover, the level of 8-hydroxy-2-deoxyguanosine (8-OHdG) in mitochondrial DNA showed a significant decrease when HCECs were subcultured with Asc-2P (Fig. 2). These results suggest that combining Asc-2P and bFGF with an atelocollagen carrier achieves successful culture of HCECs. Asc-2P may at least partly extend the lifespan of cultured HCECs through protection against oxidative DNA damage. Although the detailed mechanisms by which Asc-2P promotes cell growth remain unknown, it seems to be mediated through scavenging of reactive oxygen species (ROS) and regulating the synthesis of proteins related to cell growth. DNA is localized in both the nucleus and mitochondria. Shima et al. examined the effect of Asc-2P on the oxidative state of mitochondrial DNA, but not on that of nucleus DNA (Shima et al., 2011). As for nucleus DNA, Joyce et al. examined the relationship between oxidative DNA damage and reduced proliferation in HCECs (Joyce et al., 2009). ELISA showed that 8-OHdG levels were significantly higher in the CECs of older donors than in young donors (Joyce et al., 2009). Immunolocalization of 8-OHdG in ex vivo corneas showed more intense staining in the CECs of older donors than in CECs of young donors. They also demonstrated nuclear 8-OHdG staining was not observed in peripheral cells; however, 8-OHdG was present in a punctate cytoplasmic pattern suggesting that, in peripheral HCECs, oxidative DNA damage is located primarily in mitochondria (Joyce et al., 2009). Results of 8-OHdG immunolocalization studies in cultured HCECs paralleled those obtained in ex vivo corneas. HCECs cultured from young donors and treated with increasing concentrations of hydrogen peroxide, an inducer of oxidative stress, exhibited a dose-dependent decrease in proliferative capacity. Thus they concluded that oxidative nuclear DNA damage plays a role in the age-dependent decrease in proliferative capacity observed in HCECs (Joyce et al., 2009).

Additionally, Joyce et al. analyzed oxidative stress and DNA damage-signaling gene expression using PCR-based microarrays and demonstrated that four of 84 genes showed a statistically significant age-related difference in the expression of oxidative stress-related genes (Joyce et al., 2011). However, Western blot analysis demonstrated an age-related increase in only 2 (cytoglobin and Glutathione Peroxidase 1) of 11 proteins. Intense nuclear staining of DNA damage foci was observed in nuclei within the central endothelium of older donors, however; central endothelium from young donors consistently showed a low level of positive staining (Joyce et al., 2011). They concluded that HCECs respond to age-related increases in oxidative nuclear DNA damage by forming DNA damage repair foci; however, they do not defend against or

Table 1
Summary of culture media for human corneal endothelial cells.

Author	Basal medium	Serum	Supplements	Journal (year)
Engelmann et al.	F99 Ham's F12 & M199 (1:1 ratio)	5%	20 mg/ml ascorbic acid 20 mg/ml bovine insulin 2.5 mg/ml transferrin 0.6 ng/ml sodium selenite 10 ng/ml bFGF	In Vitro Cell Dev Biol (1989) Cornea (1995)
Miyata et al. Mimura et al.	Low-glucose DMEM	15%	2.5 mg/L amphotericin B 2.5 mg/L doxycycline 2 ng/mL bFGF	Cornea (2001) Exp Eye Res (2004a) Invest Ophthalmol Vis Sci (2004b)
Zhu and Joyce	Opti-MEM-I	8%	20 ng/ml NGF 5 ng/ml EGF 20 μg/ml ascorbic acid 200 mg/L calcium chloride 100 μg/ml pituitary extract 50 μg/ml gentamicin 1 × antibiotic/antimycotic 0.08% chondroitin sulfate	Invest Ophthalmol Vis Sci (2004)
Ishino et al.	DMEM	10%	2 ng/ml bFGF 50 U/ml penicillin 50 mg/ml streptomycin	Invest Ophthalmol Vis Sci (2004)
Li et al.	SHEM Ham's F12 & DMEM (1:1 ratio)	5%	0.5% DMSO 2 ng/ml EGF 5 mg/ml insulin 5 mg/ml transferrin 5 ng/ml selenium 0.5 mg/ml hydrocortisone 1 nM cholera toxin 50 mg/ml gentamicin 1.25 mg/ml amphotericin B	Invest Ophthalmol Vis Sci (2007)

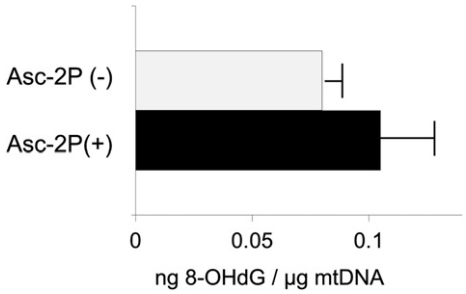

Fig. 2. Effect of Asc-2P on 8-OHdG production. HCECs from 55-year-old donor were serially subcultured six times, with or without Asc-2P, and the level of 8-OHdG in mitochondrial DNA was quantified by an ELISA. The levels showed a significant decrease when cells were subcultured with Asc-2P. Data are represented as the mean ± SD of triplicate determinations. mtDNA: mitochondrial DNA.

repair this damage by multiple oxidative stress or DNA damage-signaling genes (Joyce et al., 2011).

Treatment of cultured HCECs with H_2O_2 causes a dose-dependent decrease of cell proliferation (Joyce et al., 2009). Conversely, hypoxia stimulates the growth of various cells (Chen et al., 1995; Miyashita et al., 2007), along with a decrease of intracellular ROS (Hansen et al., 2007) and decreased expression of the negative cell cycle regulator p21 Cip1 (Lees et al., 2008). Taken together with these finding that Asc-2P suppresses intracellular ROS generation, it seems that Asc-2P may promote HCEC growth by reducing intracellular oxidative stress.

3.2. Extracellular matrices for corneal endothelial cell culture

Cell attachment and growth can be supported by seeding cells onto an artificial matrix, such as chondroitin sulfate or laminin (Engelmann et al., 1988), laminin-5 (Yamaguchi et al., 2011), the extracellular matrix secreted by bovine corneal endothelial cells (Blake et al., 1997; Miyata et al., 2001), or fibronectin plus type I collagen (Joyce and Zhu, 2004) (Table 2).

Recently, Yamaguchi and Yamagami investigated the expression of laminin-5 (LM5) and its receptors HCECs, as well as the influence of recombinant human LM5 on the adhesion, proliferation, and migration of cultured HCECs (Yamaguchi et al., 2011). They found that adult HCECs expressed the LM5 receptor ($\alpha3\beta1$ integrin), but not LM5 itself. In addition, significantly more cells were adherent to recombinant LM5-coated dishes than uncoated dishes in their cell adhesion assay, while proliferation of cultured HCECs was promoted by both LM5 and soluble LM5 in their cell proliferation assay. Furthermore, a significantly higher wound healing rate was obtained with medium containing soluble LM5 than with control medium in a wound healing assay. These findings clearly suggested that the system involving LM5 and $\alpha3\beta1$ (the receptor for LM5) is conserved in adult HCECs and that ligand-receptor interaction could promote the adhesion, migration, and proliferation of cultured HCECs.

Table 2
Summary of extracellular matrices for corneal endothelial cell culture.

Author	Extracellular matrices	Journal (year)
Engelmann et al.	Laminin and chondroitin sulfate	Invest Ophthalmol Vis Sci (1988)
Miyata et al.	Extracellular matrix secreted by bovine corneal endothelial cells	Cornea (2001)
Mimura et al.		Exp Eye Res (2004a)
		Invest Ophthalmol Vis Sci (2004b)
Joyce and Zhu	Fibronectin plus type I collagen (FNC Coating Mix®)	Cornea (2004)
Yamaguchi, Yamagami, et al.	Laminin-5	Invest Ophthalmol Vis Sci (2011)

3.3. HCEC culture

Currently, various media are used to culture HCECs, including Dulbecco's modified Eagle's medium (DMEM), Opti-MEM-I, DMEM/F12, and Ham's F12/M199, all of which achieve satisfactory results (Table 1). When Peh and associates compared these four culture media for the isolation and propagation of HCECs (Peh et al., 2011), they found that the proliferative capacity and morphology of the cells varied considerably. They concluded that HCECs cultured in Opti-MEM-I and Ham's F12/M199 had a significantly higher proliferative capacity and showed stronger expression of two markers characteristic of human corneal endothelium: Na+/K+-ATPase and ZO-1 (Peh et al., 2011). Furthermore, Engelmann's laboratory also recently evaluated the influence of five different organ culture media on corneal endothelial cell viability using five different media: HCEC growth medium (F99), standard MEM containing 2% fetal calf serum (FCS), MEM containing 5% FCS, and humanized, endothelial serum-free medium (SFM) (with and without antibiotics). They found that the number of apoptotic cells was significantly higher with MEM than with F99 or SFM, and concluded that SFM is superior to MEM for promoting HCEC survival (Jackel et al., 2011).

General culture methods for HCECs have been published by Joyce's and Amano's laboratories (Miyata et al., 2001; Chen et al., 2001; Joyce and Zhu, 2004). At Amano's laboratory, all donor corneas are obtained from the Rocky Mountain Lion's Eye Bank. Briefly, Descemet's membrane with intact endothelium is carefully dissected. After centrifugation, the strips of membrane are incubated in 0.02% ethylenediamine tetraacetic acid disodium salt solution at 37 °C for 1 h to loosen intercellular junctions. Isolated cells are plated in 6-well tissue culture plates precoated with undiluted fibronectin plus type I collagen. The cultures are then maintained in Opti-MEM-I or low-glucose DMEM containing 8% fetal bovine serum and 2 ng/mL basic fibroblast growth factor (bFGF). Joyce's laboratory uses Opti-MEM-I supplemented with 8% FBS, 5 ng/mL epidermal growth factor (EGF), 20 ng/mL nerve growth factor (NGF), 100 μg/mL bovine pituitary extract, 20 μg/mL ascorbic acid, 200 mg/mL calcium chloride, and 0.08% chondroitin sulfate for HCEC culture (Enomoto et al., 2006). Engelmann's laboratory previously immortalized HCECs by transfection with a plasmid encoding SV40 T-antigen and cultured the immortalized cells in F99 medium [a 1:1 mixture of Ham's F12 and M199] supplemented with 5% newborn calf serum, 20 μg/mL ascorbic acid, 20 μg/mL bovine insulin, 2.5 μg/mL transferrin, 0.5 ng/mL sodium selenite, and 10 ng/mL bFGF basic (Aboalchamat et al., 1999). The plates are then incubated at 37 °C in a humidified atmosphere containing 5% carbon dioxide. Once cells reach confluence, culture is continued in medium without supplements (i.e., without FGF, EGF, NGF, pituitary extract, etc.) for several days to stabilize the cell monolayer and optimally reproduce in vivo morphology (Enomoto et al., 2006). After primary cultures reached confluence, cells are subcultured at a ratio of 1:4. With this method, primary cultured HCECs usually reach confluence after 7–10 days (Fig. 3).

4. Construction of HCEC sheets

4.1. Seeding of cultured HCECs on cell carriers

During the past few decades, several laboratories have reported on the use of carriers or tissue engineering composites for construction of HCEC sheets. In the initial studies of cultured CECs, full-thickness corneal transplantation was performed in animals with

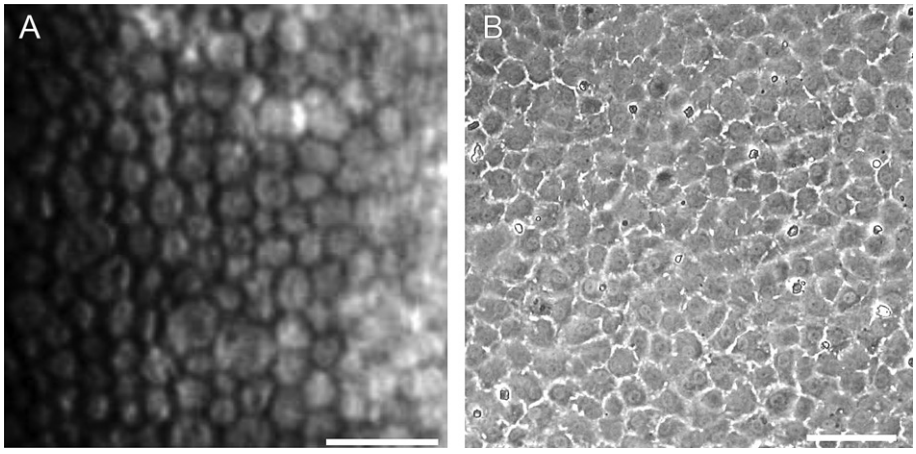

Fig. 3. Specular microscopy of a 65-year-old male patient (A) and P6 cultured human corneal endothelial cells (HCECs) derived from a 65-year-old donor (B). Confluent cells show the characteristic hexagonal shape of corneal endothelial cells. Scale bar = 100 μm.

reconstructed corneal grafts bearing cultured animal CECs seeded on Descemet's membranes from cats (Gospodarowicz et al., 1979a), rabbits (Jumblatt et al., 1978; Gospodarowicz et al., 1979b; Gospodarowicz and Greenburg, 1979), cattle (Gospodarowicz and Greenburg, 1979; Lange et al., 1993), and mice (Joo et al., 2000), or on gelatin membranes (McCulley et al., 1980; Jumblatt et al., 1980). Similar transplantation has also been performed using cultured human neonatal and infant CEC (Insler and Lopez, 1986, 1991a,b).

The origin of CEC sheets constructed from cultured cells goes back to the late 1970s. Gospodarowicz and Greenburg first introduced the method of seeding CECs onto a cell carrier (Gospodarowicz et al., 1979a, 1979b; Gospodarowicz and Greenburg, 1979). They seeded cultured bovine CECs onto bovine or rabbit corneas after removal of the endothelium. This was an epoch-making advance and the latest methods for tissue engineering of the corneal endothelium are still based on their method. Subsequently, Engelmann's laboratory developed a technique for seeding cultured HCECs onto human corneas denuded of endothelium (Aboalchamat et al., 1999; Engelmann et al., 1999; Bohnke et al., 1999).

Workers at Joyce's laboratory used a modification of the original method described by Gospodarowicz and Greenburg (Gospodarowicz et al., 1979a, 1979b; Gospodarowicz and Greenburg, 1979; Chen et al., 2001), and seeded cultured HCECs (from primary culture or passage 1) onto denuded Descemet's membrane of a second donor cornea at a density of 5×10^5 cells/mL. Then the recipient corneas were incubated in organ culture for as long as 2 weeks. Examination of HCECs in the reconstructed corneas by ZO-1 staining revealed tight junctions similar to those of in vivo corneas, and the mean endothelial cell density of the transplanted corneas was 1895 cells/mm^2 (range: 1503–2159 cells/mm^2) (Chen et al., 2001). Amano's team have also succeeded in reconstruction of human cornea using cultured HCECs and human donor Descemets' membrane denuded of endothelium (Amano, 2002, 2003) (Fig. 4).

Ishino and associates used amniotic membrane as a carrier to culture HCECs for transplantation (Ishino et al., 2004). They reported that the density of these cells grown on amniotic membranes was greater than 3000/mm^2. They transplanted cultured HCEC sheets onto rabbit corneas with PKP as follows. Rabbit corneal buttons were created by excising a 7.00-mm circle from the central cornea and Descemet's membrane with the endothelium were stripped from each button. Then a circular HCEC sheet (6.25 mm diameter) was placed on each corneal button and the buttons with HCEC sheets were grafted back onto the site of excision in the same animal and sutured with eight interrupted stitches plus a continuous suture. The transplanted corneas remained transparent for 7 days after PKP.

Amano's team have also succeed in maintaining corneal transparency during the entire observation period of 6 months in a rabbit model (Amano, 2002, 2003) and of 1 month in a nude rat model (Mimura et al., 2004a) after PKP by using cultured HCECs to construct full-thickness corneal grafts. These previous studies were based on the concept of reconstructing the full-thickness cornea by using cultured HCECs because full-thickness corneal transplantation is often performed for endothelial cell diseases such as Fuchs dystrophy and peudophakic or aphakic bullous keratopathy. However, this technique is associated with frequent complications such as high or irregular astigmatism, refractive errors, and suture-related problems. More recently, DSAEK has become a standard procedure for corneal transplantation in patients with endothelial dysfunction (Gorovoy, 2006; Koenig and Covert, 2007; Price et al., 2008; Terry et al., 2008). It achieves better postoperative visual function and reduces the risks associated with penetrating keratoplasty, such as severe astigmatism and expulsive hemorrhage. In 2004, Mimura et al. established a method of transplanting cultured HCEC sheets before DSAEK was developed (Mimura et al., 2004b).

As the cell carrier, collagen sheets (Nippi Biomatrix Research Institute, Tokyo, Japan) were employed. These sheets are composed of a network of loosely cross-linked type I collagen fibers treated with an alkaline solution, dried, and sterilized for 2 h under ultraviolet light (Stenzel et al., 1969; Hattori et al., 1999). Before use, the desiccated sheets were immersed in sterile saline for 10 min. A 6.0-mm trephine was used as the biopsy punch. Then 1.0×10^6 HCECs in 300 μL of culture medium were transferred to a collagen sheet in each well of a 96-well plate. The plates were centrifuged at 1000 rpm (176 g) for 10 min to promote attachment of cells to the sheets. After culture for 2 days, nonadherent cells and debris were removed. This method succeeded in achieving a mean endothelial cell density >3000 cells/mm^2 for cultured HCECs by improving the cell seeding technique. Adhesion of cells was promoted by centrifugation after seeding by the method of Jumblatt et al. (1978) and Engelmann et al. (1999) (Bohnke et al., 1999) with some modifications. Addition of fibronectin before cell seeding and a longer centrifugation time were found to prevent detachment of HCECs from Descemet's membrane.

After Mimura's initial report, they developed a thin human corneal stromal disc as a carrier for cultured HCEC sheets in 2009 (Honda et al., 2009). They also constructed HCEC sheets by seeding

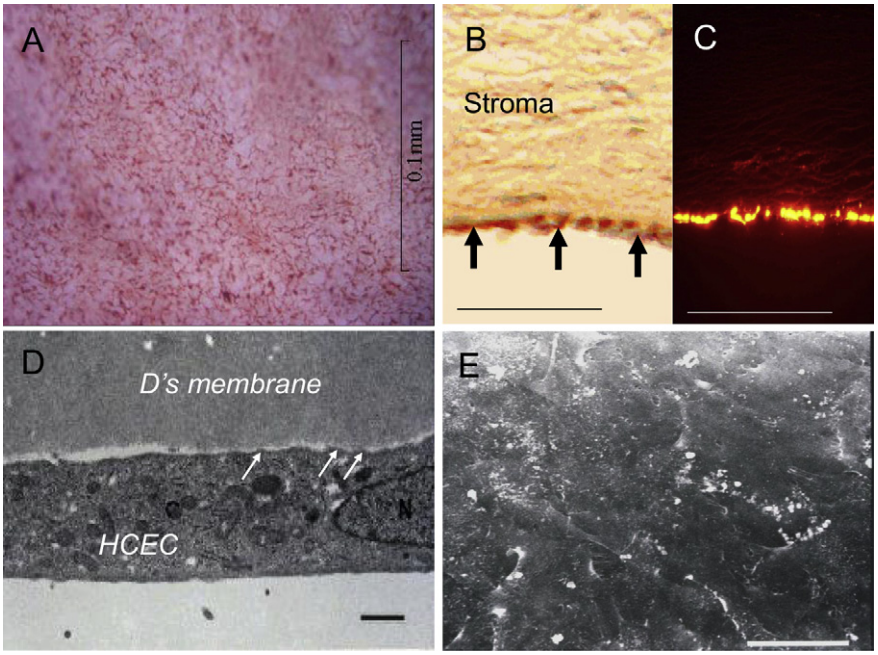

Fig. 4. Histologic examination of corneas reconstructed with cultured HCECs. Cultured HCECs were seeded on human donor Descemets' membrane denuded of endothelium and maintained in culture medium for 48 h. The adherent HCECs achieve a higher density on the human donor corneal stroma by staining with alizarin red and trypan blue (A) and form a monolayer as shown by HE staining (arrows in B). These cells are DiI-positive in cross section under a fluorescence microscope (C). Transmission electron micrograph demonstrates attachment of HCEC to the Descemet's membrane of donor cornea by hemidesmosomes (arrows in D). Under the scanning electron microscope, a typical polygonal structure of HCECs can be visualized (E). Scale bars = 100 μm (A, B, and C) and 0.1 μm (D and E).

cultured cells onto various carriers such as decellularized thin porcine corneal stroma and human amniotic membrane (Fig. 5). These new HCEC sheets showed similar morphologic features to the collagen-based HCEC sheets.

Nishida's group reported a monolayer HCEC sheet that was grown in temperature-responsive culture dishes (Sumide et al., 2006). They initially cultured HCECs in type IV collagen-coated dishes and seeded the cultured cells into temperature-responsive culture dishes after several passages. On scanning electron microscopy, the cells in their HCEC sheets were mainly hexagonal in shape with numerous microvilli and cilia, similar to the cells of the native corneal endothelium. Lai and associates cultured HCECs on thermoresponsive poly (N-isopropylacrylamide) (PNIPAAm) and seeded the cultured cells onto gelatin hydrogel discs to create HCEC sheets (Lai et al., 2007). Choi and associates used decellularized thin-layer human corneal stroma as a carrier (Choi et al., 2010). Liang and associates also developed a chitosan-based membrane made of hydroxyethyl chitosan, gelatin, and chondroitin sulfate as a new carrier for cultured HCEC sheets (Liang et al., 2011). Additionally, Nishida and Tabata's group recently developed gelatin hydrogel as a carrier for the construction of HCEC sheets (Watanabe et al., 2011), and they demonstrated that HCECs cultured on these sheets showed normal expression of ZO-1, Na^+–K^+ ATPase, and N-cadherin. These newer materials could be very attractive candidates for future use in HCEC tissue regeneration.

4.2. Pump function of HCEC sheets

Corneal hydration and transparency depends primarily on the transport of sodium and bicarbonate ions driven by the Na+/K+-adenosine triphosphatase (Na+/K+-ATPase) pump (Brown and Hedbys, 1965; Hodson, 1971; Huff and Green, 1981; Fischbarg et al., 1985). Therefore, HCEC sheets must possess transport activity to maintain corneal transparency after transplantation. When the pump function of HCECs grown on collagen sheets was measured in an Ussing chamber by the method reported previously with some modifications (Hodson and Wigham, 1983; Wigham and Hodson, 1981; Wigham et al., 2000), the mean potential difference of the HCEC sheets at 1, 5, and 10 min was respectively 85%, 80%, and 95% of that for human donor corneas (Fig. 6A). In addition, the average short-circuit current of the HCEC sheets at 1, 5, and 10 min was respectively 76%, 78%, and 82% of that for human donor corneas denuded of epithelium. Furthermore, the potential difference and short-circuit currents of the HCEC sheets and human donor corneas denuded of epithelium and endothelium were both 0 mV and 0 A at each time of assessment (Fig. 6A and B). After the Na^+, K^+-ATPase inhibitor ouabain was added, the potential difference and short-circuit currents reached 0 mV for all test samples within 5 min. These results indicate that the pump function of HCEC sheets (mainly depending on Na^+, K^+-ATPase) was satisfactory.

5. Transplantation of DSAEK grafts in a rabbit model

Mimura et al. described the first application of cultured HCEC sheets for DSAEK in 2004 (Mimura et al., 2004b). In that study, they used collagen sheets as the carrier for cultured HCECs. In their second study, DSAEK grafts were produced by seeding cultured HCEC suspensions onto thin human corneal stromal discs (Honda et al., 2009). When these DSAEK grafts were transplanted onto rabbit corneas, the grafted HCECs maintained a similar morphology to HCECs in vivo and reduced corneal edema.

Several other groups have also reported on DSAEK grafts using cultured CECs (Table 3). Lai and associated transplanted HCEC sheets cultured on PNIPAAm and gelatin-based material into the eyes of rabbits, and found that corneal transparency was restored within 2 weeks after surgery (Lai et al., 2007). Koizumi and associates created monkey CEC sheets by culturing monkey CECs on type I collagen for 4 weeks (Koizumi et al., 2007, 2008), and then transplanted the CEC sheets into the eyes of monkeys by the DSAEK technique. The transplanted sheets became detached from the host

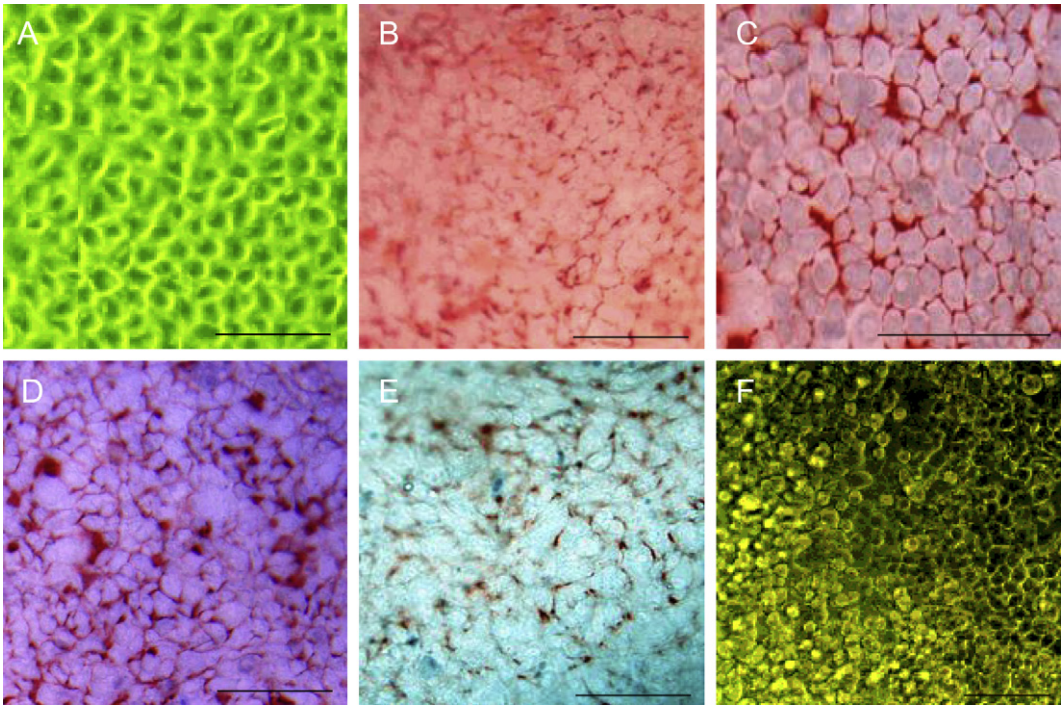

Fig. 5. Cultured human corneal endothelial cells (HCECs) (A) and bioengineering of corneal endothelium using cultured HCECs (B–F). (A) Confluent cultured passage 6 HCECs from a 62-year-old donor show the characteristic hexagonal shape of corneal endothelial cells. (B–F) Corneal endothelium was reconstructed by seeding cultured HCECs on various carriers to make grafts for penetrating keratoplasty (PKP) (B, C) or Descemet stripping with automated endothelial keratoplasty (DSAEK) (D, E, F). The endothelial surface was observed under a light microscope after culture for 24 h. (B) Cultured HCECs were seeded on human donor corneal stroma denuded of endothelium for PKP grafts. (C) Cultured HCECs were seeded on rat cornea denuded of endothelium for PKP grafts in rat corneal transplantation model. (D) Cultured HCECs were seeded on decellularized thin porcine corneal stroma for DSAEK grafts. (E) Cultured HCECs were seeded on human amniotic membrane for DSAEK grafts. (F) Cultured HCECs were seeded on collagen sheets for DSAEK grafts. Scale bars = 100 μm.

corneas by one week after grafting, but the corneas recovered clarity by six months after transplantation of these monkey CEC sheets.

Mimura et al. introduced DSAEK model using cultured HCEC sheets and describe the observations made after transplantation of DSAEK grafts (Mimura et al., 2004b; Honda et al., 2009). For the first study of DSAEK grafts using cultured HCECs, a 6-mm sclerocorneal incision centered at 12 o'clock was made with a slit knife, a circular Descemetorhexis (6.0 mm in diameter) was created in the center of the cornea with a 30-gauge needle, and Descemet's membrane was removed from the anterior chamber in rabbits. A circular HCEC sheet (with the HCEC side up) was brought into the anterior chamber and then was fixed to the posterior stroma after Descemet's membrane had been stripped off. If the sheet proved difficult to attach, an air bubble was injected into the anterior chamber. The sclerocorneal wound was closed with two or three interrupted sutures of 10-0 nylon. This transplantation technique is similar to the clinical DSAEK procedure. The rabbits were divided into a DSAEK group (peeling of Descemet's membrane and transplantation of an HCEC sheet) and a control group (peeling of Descemet's membrane only), with each group comprising four rabbits (four eyes).

Corneal edema decreased much sooner after HCEC sheet transplantation in the DSAEK group than in the control group. In

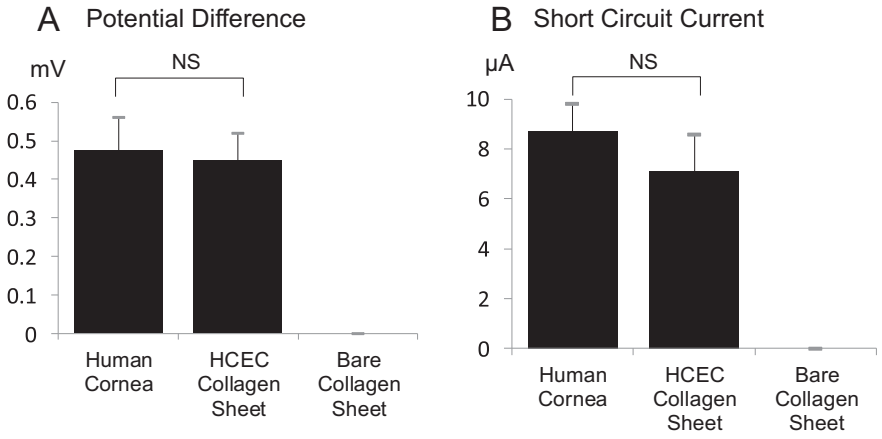

Fig. 6. Pump function of reconstructed HCEC sheets (modified from reference (Mimura et al., 2004b) with permission). The potential difference (A) and short-circuit current (B) of human donor corneas ($n = 4$), cultured HCEC collagen sheets ($n = 4$), and bare collagen sheets ($n = 4$) was measured by an Ussing chamber. Data are shown as the mean ± SD. Ns = not significant (human donor cornea vs. cultured HCEC collagen sheet, unpaired *t*-test).

Table 3
Summary of transplantation technique of cultured corneal endothelial cell sheets in animal models.

Author	Species of cultured CEC	Cell carrier	Host animal	Transplantation technique	Journal (year)
Mimura	Human	Collagen	Rabbit	DSAEK	Invest Ophthalmol Vis Sci (2004)
Ishino	Human	Amniotic membrane	Rabbit	PKP	Invest Ophthalmol Vis Sci (2004)
Sumide	Human	PNIPAAm	Rabbit	PKP	FASEB J (2006)
Lai	Human	PNIPAAm and gelatin	Rabbit	DSAEK	Transplantation (2007)
Koizumi	Monkey	Collagen	Monkey	DSAEK	Invest Ophthalmol Vis Sci (2007)
Honda	Human	Thin human corneal stromal disc	Rabbit	DSAEK	Arch Ophthalmol (2009)

CEC: Corneal endothelial cell, PKP: Penetrating keratoplasty, DSAEK: Descemet stripping automated endothelial keratoplasty.

the control group, the mean corneal thickness was approximately 1000 μm throughout the 28-day observation period. In contrast, it decreased rapidly in the DSAEK group, and the cornea was significantly thinner than in the control group at 1 ($P < 0.05$, unpaired t-test), 3, 7, 14, 21, and 28 days ($P < 0.001$, unpaired t-test) after surgery. Fig. 7 shows representative anterior segment photographs from each group. The control group cornea is opaque with severe stromal edema while the DSAEK group cornea is clear and has no stromal edema on day 28 after transplantation of a cultured HCEC sheet (Fig. 7).

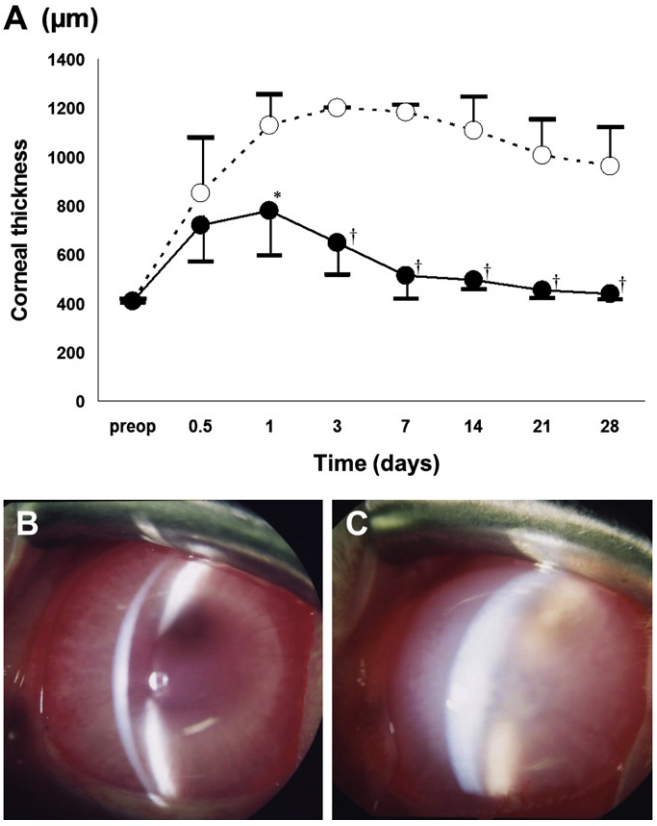

Fig. 7. Central corneal thickness (A) and anterior segment photographs (B, C) after transplantation of DSAEK grafts constructed with a collagen sheet and cultured HCECs (modified from reference (Mimura et al., 2004b) with permission). DSAEK grafts created using cultured HCECs and collagen sheets (DSAEK group) or bare collagen sheets (control group) were transplanted onto rabbit corneas after stripping of Descemet's membrane. There was a significant difference of corneal thickness between the DSAEK and control groups on days 1, 3, 7, 14, 21, and 28 (unpaired t-test, $^{*}p < 0.05$ and $\dagger p < 0.01$). Representative anterior segment photographs obtained with a slit-lamp microscope at 28 days after surgery show a thin cornea without stromal edema in the DSAEK group (B), while severe corneal edema is obvious in the control group (C).

Fluorescence microscopy of whole mounted corneas showed DiI-positive cells localized on the transplanted collagen sheet and a clear margin of the sheet at 28 days after transplantation. HCECs cultured on the collagen sheets had a fairly regular morphology and well-defined boundaries. After transplantation to the posterior surface of the cornea, most of the cells on the collagen sheets were DiI-positive in the DSAEK group (Mimura et al., 2004b). Since endocytosed DiI cannot be transferred to adjacent cells (Horan et al., 1990), this finding suggested that cultured HCECs remained on the sheets. The endothelial cell density of the grafts in the DSAEK group was around 2500 cells/mm^2 at 28 days after surgery. In contrast, no CECs were detected on the stroma at the site of Descemetorhexis in the control group.

A study of DSAEK grafting with thin human corneal stromal discs as the carrier for cultured HCECs yielded similar results to those of Honda's study (Honda et al., 2009). Mimura et al. also transplanted DSAEK graft made from HCECs cultured on human amniotic membrane into rabbit eyes (Fig. 8), and this DSAEK model also showed rapid recovery of corneal edema without side effect such as increased intraocular pressure or graft rejection. In these various DSAEK models, HCEC grafts remained transparent for 1 month after surgery and the transplanted HCEC sheets were significantly thinner than the corneas of the untransplanted control group. These results suggest the feasibility of performing corneal reconstruction with HCEC sheets derived from adult donor corneas.

6. Immunologic mechanisms of corneal allografts after transplantation

The anterior chamber of the eye is an immune-privileged site and anterior chamber-associated immune deviation (ACAID) allows the long-term acceptance and survival of histoincompatible tissue grafts that would be rejected if transplanted to other sites (Streilein and Niederkorn, 1981, Streilein et al., 1996). Mimura et al. reported no evidence of an inflammatory reaction, such as massive cell infiltration, keratic precipitates, or fibrin deposition, was detected in the anterior chamber by slit lamp microscopy after HCEC sheets transplantation, indicating that there was no notable acute rejection (Mimura et al., 2004b). When HCEC sheets are grafted, the transplanted HCECs face the anterior chamber may induce ACAID, thereby avoiding rejection, as evidenced by the lack of any immune reaction at one month after human to rabbit HCEC sheet (Mimura et al., 2004b). Another possible reason is that the collagen sheet does not permit cell infiltration.

Recently, Hayashi et al. investigated the immunologic features after cultured CEC transplantation in a murine model (Hayashi et al., 2009). They used a mouse CEC deficiency model for allo-CEC transplantation and found that chimeric CEC allografts composed of cultured allogeneic CECs did not provoke rejection reaction, donor-specific delayed hypersensitivity (DTH), or mixed lymphocyte reactions, unlike the high rejection rate that occurred in full-thickness corneal allografts. On the contrary, BALB/c mouse donor corneas (allogeneic epithelium and stroma) reconstituted with immortalized C3H/HeN (C3H) mouse (CECs) were swiftly rejected when transplanted into C3H recipients. These findings indicate that CEC is not an inducer of allograft rejection and suggest that CEC transplantation has advantages over conventional full-thickness corneal transplantation.

Several possible mechanisms have been proposed to explain the acceptance of the CEC allografts. The induction of active immunosuppression including the induction of regulatory T cells, the

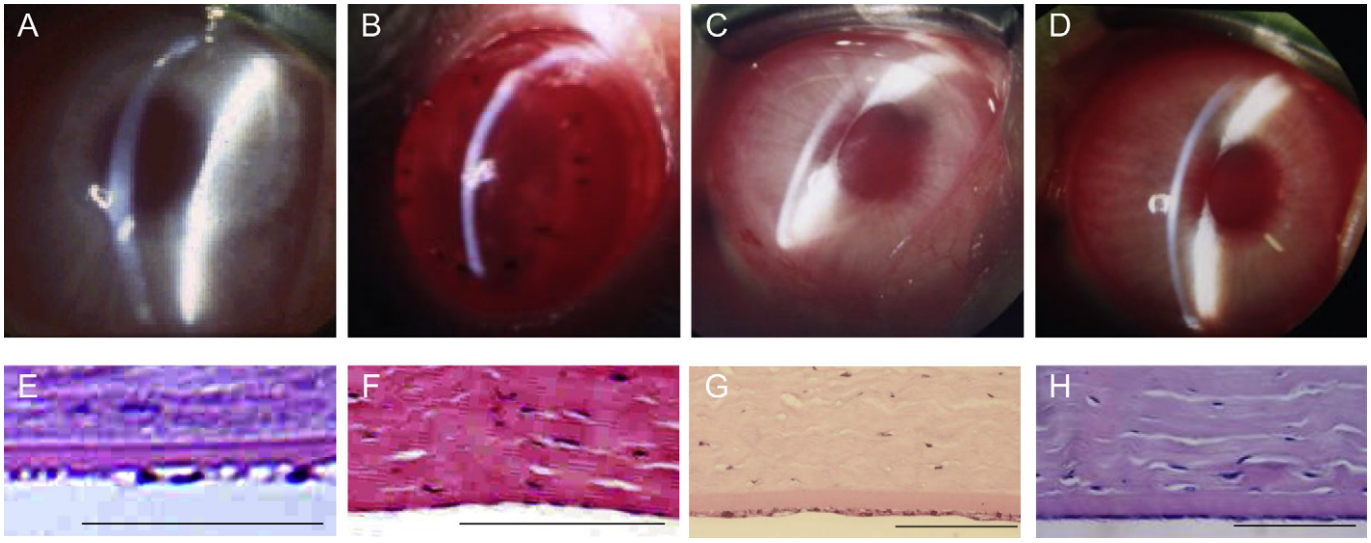

Fig. 8. Slit-lamp examination (A–D) and histological examination of the corneas (E–H) after transplantation of bioengineered corneal endothelium created by using cultured HCECs and various carriers or after transplantation of cultured HCECs into the anterior chamber (modified from reference (Mimura et al., 2004a, 2005a) with permission). (A, E) Cultured HCECs were seeded onto rabbit corneal stroma denuded of endothelium and then transplanted onto rabbit corneas after standard PKP. Appearance at 6 months postoperatively. (B, F) Cultured HCECs were seeded onto rat corneal stroma denuded of endothelium and transplanted onto rat corneas after PKP. Appearance at 1 month postoperatively. (C, G) Bioengineered DSAEK grafts created with cultured HCECs and human amniotic membrane were transplanted onto rabbit corneas after stripping of Descemet's membrane. Appearance at 1 month postoperatively. (D, H) Cultured HCECs were injected into the anterior chamber and attached to the surface of the cornea after it was denuded of endothelium in rabbits. Appearance at 12 months postoperatively. Corneas transplanted with cultured HCECs remained clear (A–D) and HCECs were present on the posterior corneal surface (E–G) in all cultured HCEC transplantation models. Scale bars = 50 μm.

induction of T-cell anergy, and immunologic ignorance of draining lymph node T-inducer priming cells (Sonoda et al., 2002; Akl et al., 2005; Kang et al., 2007; Tao and Hancock, 2007; Yang, 2008). Active immunosuppression is induced in mice that show long-term acceptance of full-thickness corneal transplantation (Sonoda and Streilein, 1993; Yamada et al., 2005). However, CEC allografts failed to induce active immunosuppression, as evidenced by adoptive transfer of splenocytes from mice that had accepted chimeric CEC allografts and regraft challenge in CEC allograft-accepted mice. Niederkorn's group and Amano's group demonstrated the induction of ACAID after the injection of CECs into the anterior chamber (Niederkorn and Mellon, 1996; Hayashi et al., 2009). However, ACAID was not induced after CEC allograft transplantation (Hayashi et al., 2009). These findings suggest that CEC graft survival without rejection did not result from the induction of immunosuppression or T-cell anergy. Given that subcutaneous injection of splenocytes syngeneic to the chimeric CEC allografts did not increase the rejection rates of CEC allografts while showing a high systemic DTH response to the same antigen, immunologic ignorance seems to be the main reason for the rejection-free acceptance of chimeric CEC allografts.

7. Human corneal endothelial precursor cells

7.1. Overview of stem cells or precursors

Stem cells or progenitor cells are defined by a capacity for self-renewal and the ability to generate different types of cells (multipotentiality) that are involved in the formation of mature tissues. In contrast, precursor cells are unipotential cells with limited proliferative capacity. Regenerative stem cells or precursors can be detected by the sphere-forming assay in various adult tissues, including the central nervous system (Nunes et al., 2003), bone marrow (Krause et al., 2001), skin (Toma et al., 2001; Kawase et al., 2004), retina (Coles et al., 2004), corneal epithelium (Yokoo et al., 2005; Mimura et al., 2010a), corneal stroma (Uchida et al., 2005; Amano et al., 2006; Yamagami et al., 2007; Mimura et al., 2008a, 2008b), and corneal endothelium (Yokoo et al., 2005; Mimura et al., 2005a, 2005b, 2005c, 2007, 2010b; Amano et al., 2006; Yamagami et al., 2006, 2007). Despite the successful isolation and characterization of stem cells from various tissues, relatively few animal studies have been done to investigate the efficacy of stem cell transplantation. A three-dimensional carrier that maintains cell-to-cell interactions is indispensable for tissue engineering using stem cells, but the resulting structural complexity does not allow us to easily perform investigations of stem cell transplantation.

In 2005, the precursors with the propensity to develop into corneal endothelial-like cells have been isolated from the CE of human donor corneas (Yokoo et al., 2005). It has been also demonstrated that cultured human corneal endothelial cells (HCECs) and rabbit CE-derived precursors are an effective cell source for treating corneal endothelial defects in a rabbit model (Mimura et al., 2005a, 2005b). Because the number of CEC precursors that can be isolated from a native cornea is insufficient for corneal transplantation, establishment of a method for the mass production of precursor cells is required before CEC transplantation can be employed clinically. Topics covered in this chapter include the recent work in the fields of regenerative medicine and tissue engineering for the CE using bipotential precursor cells.

7.2. Isolation of sphere colonies from human corneal endothelium

CE precursors can be isolated by the sphere-forming assay from human donor corneas (Yokoo et al., 2005). After incubation for 5 days, small floating spheres form. These spheres grow larger after 10 days (Fig. 9A). Most of the cells in each sphere on day 10 could be labeled with BrdU (Fig. 9B), indicating that the spheres contained proliferating cells. These results suggested that the sphere colonies arose from single isolated HCECs and that the sphere-forming cells possess the capacity to proliferate. 257 ± 83 spheres (mean ± SD, $n = 8$) are generated per dish (50,000 cells). In a typical case, 2.5 ± 104 cells are isolated from a 10-mm piece of corneal tissue, generating approximately 130 spheres after 10 days. These spheres have a diameter of 88.3 ± 15.9 μm (mean ± SD, $n = 35$). The

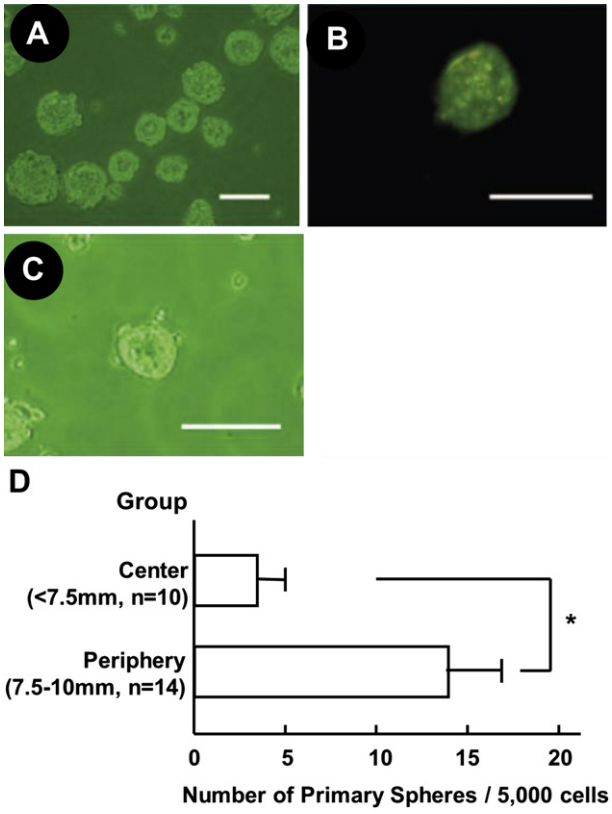

Fig. 9. Sphere formation from donor human corneal endothelium. (A) Floating spheres formed by sphere-forming culture on day 10. (B) Each sphere colony was labeled with BrdU on day 10. (C) Secondary spheres generated after the dissociation of primary spheres. (D) The number of primary spheres obtained was compared between the peripheral (7.5–10.0 mm in diameter) and central regions (7.5 mm in diameter) of the cornea. The number of sphere colonies obtained from the peripheral cornea ($n = 14$) after 10 days of culture was significantly higher than that obtained from the central cornea ($n = 10$) (unpaired t-test). This experiment was repeated 3 times using different donor corneas, and representative data are shown as the mean ± SD. $^*P < 0.0001$. These figures were modified from Yokoo et al. (2005) and Yamagami et al. (2007) with permission.

replating efficiency shows a dramatic decline between primary and secondary sphere colonies. When the primary spheres are trypsinized and incubated in serum-free floating culture, secondary colonies are generated (Fig. 9C) at a level of approximately 15 ± 1 ($n = 3$) per dish of 10,000 cells. This suggests that HCECs have the capacity for self-renewal and formation of sphere colonies, but this capacity is limited.

7.3. Distribution of precursors derived from corneal endothelium

Regarding the distribution of ASCs or precursors in ocular tissues, mouse and human retinal ASCs were reported to be located at the ciliary margin (Tropepe et al., 2000; Coles et al., 2004). In animals and humans, various parts of the conjunctiva have been suggested as ASC-producing areas, including the limbus, bulbar conjunctiva, fornix, palpebral conjunctiva, and mucocutaneous junction (Wei et al., 1993, 1995; Pe'er et al., 1996; Lavker et al., 1998; Pellegrini et al., 1999; Wirtschafter et al., 1999; Chen et al., 2003; Nagasaki and Zhao, 2005). In the cornea, epithelial precursors are restricted to sites outside the clear cornea (i.e., the corneal limbus), but limbal ASC-specific markers have not yet been identified (Kinoshita et al., 2001; Grueterich et al., 2003; Lavker et al., 2004). In rabbit, the peripheral endothelium contains more precursors with a stronger self-renewal capacity relative to the central area (Mimura et al., 2005b). In humans, an increased density of HCE cells has been detected in the paracentral and peripheral areas of the cornea by specular microscopy and histological examination of donor corneas (Schimmelpfennig, 1984; Amann et al., 2003). p53 is a negative regulator of cell division that is strongly expressed by central HCE cells (Paull and Whikehart, 2005). The peripheral HCE cell density was reported to be lower than the central HCE cell density (Binder et al., 1979). These findings suggest that a greater number of HCE cell precursors reside in the corneal periphery and proliferate slowly in vivo.

Human corneal endothelium also contains more precursors in the peripheral area compared with the central area as shown in Fig. 9D (Yamagami et al., 2007). The rate of sphere formation by HCECs from the peripheral cornea is approximately 4 times that for HCECs from the central cornea in repeated experiments.

It has generally been accepted that human CE does not proliferate after birth, but some previous reports suggest that the CE may undergo slow proliferation in vivo. In 2003, Amann et al. demonstrated that paracentral and peripheral HCECs exist at a higher density than central HCECs by specular microscopy and histological observation of donor corneas (Amann et al., 2003). The presence of slowly proliferating HCEC precursors in the peripheral cornea could explain this higher cell density at the periphery. Otherwise, the cell density should be uniform throughout the corneal endothelium, because it tends to equalize over time. Another suggestive point is the outcome of Sato's method of anterior–posterior refractive surgery that involves making multiple peripheral and midperipheral incisions in the endothelium and stromal layer from the anterior chamber to treat myopia (Kanai et al., 1982; Kawano et al., 2003). This type of radial keratotomy performed via the anterior chamber leads to a decrease of HCECs many years later, possibly as a result of the corneal incisions causing more rapid cell loss than would occur with normal aging (Kanai et al., 1982; Kawano et al., 2003). It is possible that direct damage to HCEC precursors slows their proliferation, so that replacement of CECs decreases. The third point to consider is the outcome of corneal transplantation for various conditions associated with damage to the cornea, such as bullous keratopathy, keratoconus, and corneal leukoma. In hosts who retain their peripheral CE, such as patients with keratoconus, the grafts survive for much longer than in hosts with loss of the peripheral endothelium, such as patients with bullous keratopathy (Williams et al., 1992; Boisjoly et al., 1993; Yamagami et al., 1996). Keratoconus patients are typically younger than those with bullous keratopathy, so it could be suggested that their peripheral endothelium has greater proliferative potential because of this age difference, but differentiation of CEC precursors from the host cornea augmenting viable cells from the graft may be another reason for the longer survival of grafts after transplantation for keratoconus compared with bullous keratopathy. Therefore, when full-thickness corneal transplantation is done, a larger graft may be preferred for eyes with bullous keratopathy because it can supply more HCEC precursors, whereas a smaller graft may allow the optimum use of host-derived HCEC precursors in patients with keratoconus.

7.4. Characterization and proliferative capacity of HCE precursors

Individual spheres contain cells that express markers of the mesenchymal and neural lineages as shown in Fig. 10. HCE precursors in the spheres show immunoreactivity for nestin and for α-SMA, but not for p75 NTR. Spheres are positive for an immature neuronal marker (β3-tubulin) and an astroglial marker (GFAP), but not a mature neuronal marker (NFM), an oligodendroglial marker (O4), or a peripheral nerve neuronal marker (peripherin). These findings indicate that spheres isolated from human donor CE

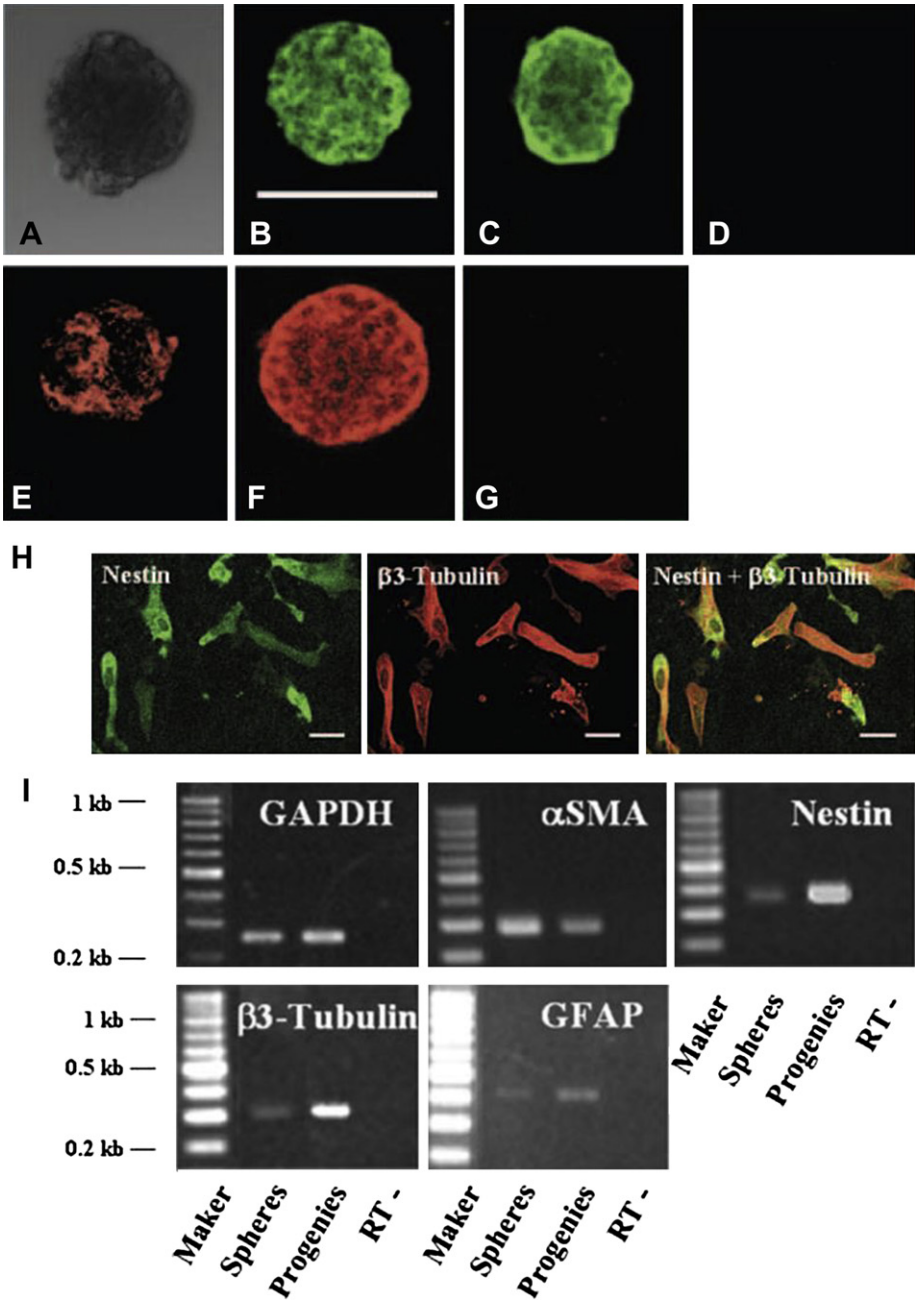

Fig. 10. Immunocytochemistry (A–H) and RT-PCR analysis (I) of sphere colonies and their progeny. (A) Bright-field image of a typical sphere colony. (B) Immunostaining of the entire sphere on day 10 identifies cells expressing nestin, a marker of immature cells. (C–G) Spheres show immunostaining for a mesenchymal myofibroblast marker (α-SMA, C), an immature neuronal marker (β3-tubulin, E), and an astroglial cell marker (GFAP, F). Sphere colonies derived from HCECs are not stained by nonimmunized mouse IgG (D) or normal rabbit serum (G). Differentiated cells derived from primary spheres are double immunostained by nestin and β3-tubulin, indicating that the colonies contain immature (undifferentiated) cells. (I) RT-PCR analysis of cells from spheres and their progeny. GAPDH gene expression is detected in the sphere colonies and their progeny, but not when reverse transcription is omitted. Nestin, α-SMA, β3-tubulin, and GFAP genes are detected in both spheres and their progeny, but not when total RNA is processed without reverse transcription. Scale bars = 100 μm (A–G) or 200 μm (H). Figures are modified from Yokoo et al. (2005) with permission.

contain bipotential precursors that are capable of undergoing differentiation into mesenchymal cells and neuronal cells.

Secondary spheres can be generated from the dissociated primary spheres, but the yield of secondary sphere colonies is lower than after primary culture (Yokoo et al., 2005). Although self-renewal potential is indicated by the ability of cells from individual primary spheres to form secondary spheres, this potential is limited, as evidenced by the failure of sphere formation at the third passage. These results indicate that the precursor cells have a limited proliferative capacity. Photographs of representative secondary spheres are shown in Fig. 9C.

7.5. Differentiation of HCE precursors

The progeny from primary HCE spheres express nestin and β3-tublin on immunocytochemistry and RT-PCR as shown in Fig. 10. Adherent differentiated cells from the sphere colonies have an HCEC-like hexagonal shape and satisfactory transport activity that is essential in HCECs (Yokoo et al., 2005). These findings indicated that spheres isolated from human CE contain bipotential precursors, yielding progeny that display the morphologic characteristics of HCECs. Taken together, these results suggest that precursors from the CE remain close to the tissue of origin and

undergo differentiation into CECs. Because precursors should ideally differentiate efficiently to produce their tissue of origin, precursors obtained from the CE may be more appropriate for tissue regeneration or cell transplantation than those derived from the multipotential stem cells.

8. Selective isolation of precursors from cultured HCECs

8.1. Concept of isolation of young cells from cultured cells

The sphere-forming assay is a representative technique for the isolation of potential stem cells or precursor cells. With this technique, isolated single cells are allowed to proliferate in floating culture and form cell clusters under clonogenic conditions. This technique was initially devised to enrich neural stem cells. As mentioned in Sections 7.1–7.4, precursor cells can be isolated from human donor corneas (Yokoo et al., 2005; Yamagami et al., 2007). However, the number of precursors that can be isolated from a cornea is insufficient for corneal endothelial regeneration, so establishment of a mass production method for precursor cells is needed before clinical application can be attempted. Topics covered in this chapter include the isolation of spheres from cultured HCECs and the effect of injecting these spheres into the anterior chamber.

8.2. Isolation and characterization of precursors from cultured HCECs

Cultured HCE can form primary and secondary sphere colonies as shown in Fig. 11 (Mimura et al., 2010b). The progeny shows an HCE-like hexagonal shape and has adequate transport activity. The findings suggest that culture of HCE can promote mass production of HCE precursors, determined by sphere-forming assay.

Spheres derived from donor CE express an immature cell marker (nestin), an immature neuronal marker (β-III tubulin), and a mature glial cell marker (GFAP), while their progeny expressed β-III tubulin and nestin, but not GFAP. In contrast, the spheres and progeny obtained from cultured HCECs do not express neuronal markers and show decreased expression of immature cell markers. These findings suggest that the precursors are close in nature to the original tissue and underwent differentiation during culture. Thus, precursors obtained from cultured HCECs may be a more appropriate cell source than cells from donor CE, because precursors that efficiently differentiate into the tissue of origin are ideal for tissue regeneration or cell transplantation.

8.3. Cellular senescence in precursors isolated from cultured HCECs

Cellular senescence is characterized by enlargement of cells, inability to regenerate, and an increase of SA-b-gal activity, and is induced by critical telomere shortening (Wang et al., 2003). Precursor cells isolated from cultured HCECs by the sphere-forming assay have longer telomeres and higher telomerase activity than the original cells (Mimura et al., 2010b). The progeny of the spheres showed little staining level for the cell senescence marker SA-b-Gal and high expression of the immature cell marker nestin. They also had a regular morphology and grew at a higher density with greater BrdU incorporation than passaged cells derived from the same parental cells. These findings indicate that the sphere-forming assay is an easy and useful method of isolating young (precursor) cells from various other cells.

8.4. Telomere length in precursors isolated from cultured HCECs

The telomere length of human CEC in vivo has been reported to be 11–12.5 kb (Egan et al., 1998), and there was no significant

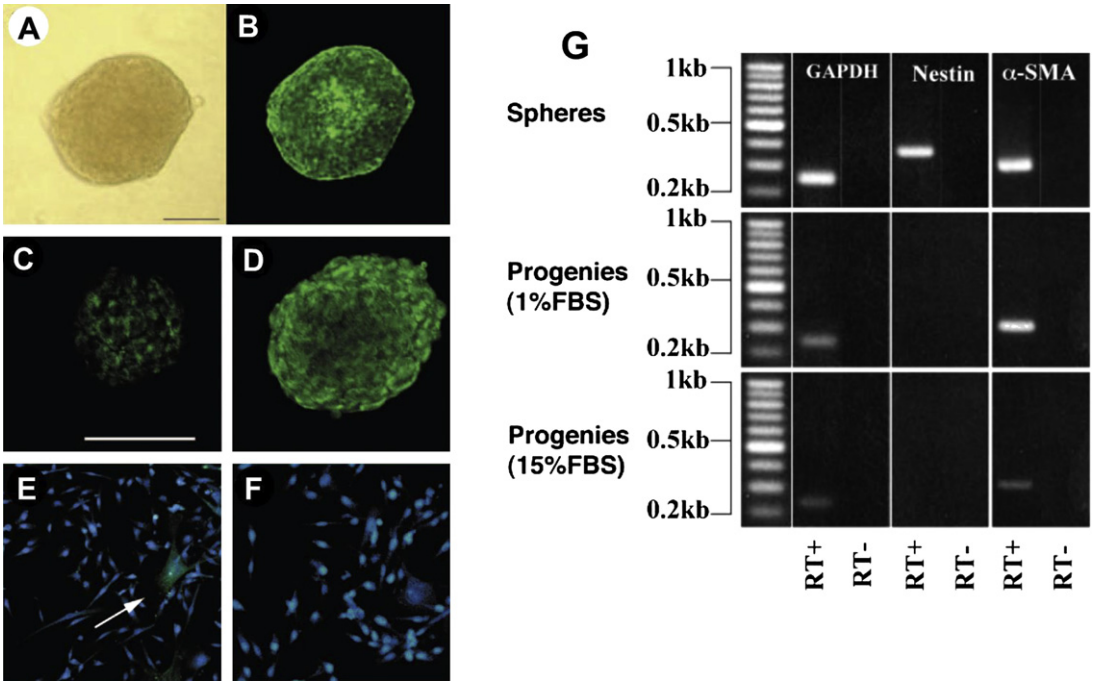

Fig. 11. Immunocytochemistry (A–F) and RT-PCR analysis (G) of sphere colonies derived from cultured HCECs were their progeny. (A) A representative day 7 sphere. (B) Cells in a sphere colony labeled by BrdU on day 7. Scale bar = 50 μm (C–F) A day 7 sphere shows staining for nestin (C) and α-SMA (D). Less than 5% of the sphere progeny cells were stained by the mesenchymal cell marker α-SMA (E, arrow). There is no staining by control IgG (F). Scale bar = 100 μm. (G) RT-PCR of spheres and progeny. cDNA was obtained from spheres and from their progeny cultured in 1% FBS or 15% FBS. GAPDH was detected in all samples, except those reacted without reverse transcriptase. Nestin mRNA expression was detected in cultured spheres, but not in their progeny cultured in either 1% or 15% FBS. Both the spheres and progeny were positive for α-SMA mRNA. Figures are modified from Mimura et al. (2005c) with permission.

difference in telomere length between the central and peripheral areas of human donor corneas (Konomi and Joyce, 2007). Normally, a small amount of telomeric DNA (~50–100 bp) is lost with each human somatic cell division (Allsopp et al., 1992). The average telomere length of precursor cells within spheres (11.4 kb) is longer than that of P7-cultured human CECs (9.5 kb), suggesting that precursor cells within sphere possess stronger replicative capacity of approximately 19–38 divisions in culture compared with P7-cultured human CECs as shown in Fig. 12. Cells isolated from spheres have longer telomeres than the original cells, but the mechanism by which telomeres are elongated in the sphere-derived cells is unknown. It was reported that overexpression of telomerase leads to elongation of telomeres in human bone marrow stromal cells (Simonsen et al., 2002), whereas telomerase overexpression does not prevent proliferation-associated telomere shortening in human hematopoietic cells (Wang et al., 2005). Because only 0.5% of cultured human CECs are sphere-forming cells, it may be that cells with long telomeres are selectively isolated by this assay and senescent cells with shorter telomere are eliminated. However, there is the possibility that temporary upregulation of telomerase elongates the telomeres to some extent during culture.

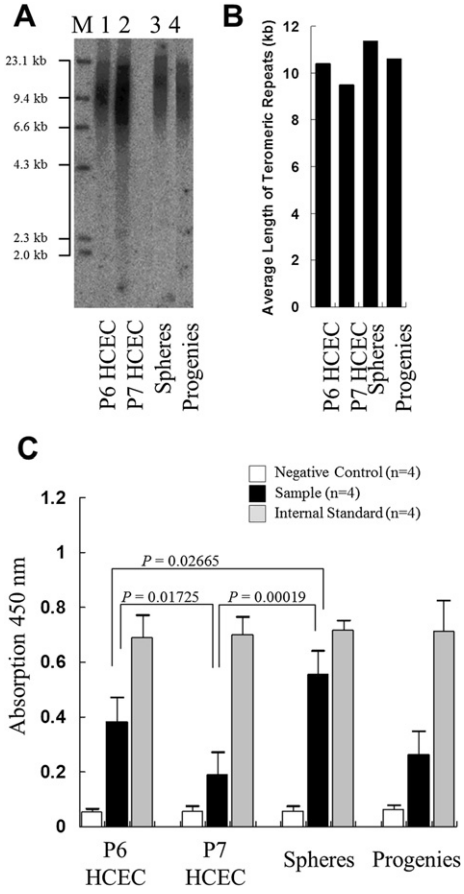

Fig. 12. Southern blot analysis of the length of telomeric repeats (A), diagram showing the average length of telomeres (B), and telomerase activity in P6- and P7-cultured human CEC, spheres derived from P6-cultured human CEC, and progeny of the spheres (C). Cells are derived from a 41-year-old donor. The average length of telomeres in the precursors from spheres (11.4 kb) and their progeny (10.6 kb) was longer than that of P7-cultured human CECs (9.5 kb). Average telomere length is shown as bars (B). Telomerase activity is significantly lower in P7-cultured CECs than in P6-cultured CECs (C). The cells of spheres derived from P6 CECs show significantly higher telomerase activity than P6-cultured CECs. Heat-treated samples were employed as the negative control, and internal standards provided were the positive control. Error bars show the standard deviation ($n = 4$ each). One-way analysis of variance and Scheffe's multiple comparison tests were used to compare mean values. Figures are modified from Mimura et al. (2010b) with permission.

8.5. Telomerase activity in precursors isolated from cultured HCECs

Telomerase activity is high in germ cells and decreases in the sperm and ova (Hiyama and Hiyama, 2007). In almost all somatic cells, telomerase activity is undetectable or very low. In human CECs, the telomerase was localized in the peripheral CECs (McGowan et al., 2007), but the central CECs show no telomerase activity (Whikehart et al., 2005). Adult stem cells also show undetectable or low telomerase activity regardless of their proliferative capacity, whereas committed precursors with high proliferative activity have comparatively high levels of telomerase activity (Chiu et al., 1996; Hiyama and Hiyama, 2007). However, their telomerase activity is still not adequate for maintenance of telomere length (Hiyama and Hiyama, 2007) Spheres derived from cultured HCECs (Mimura et al., 2005b, 2005c, 2007) shows higher telomerase activity that was consistent with the characteristics of lineage-committed precursors rather than adult stem cells with lower telomerase activity (Fig. 12C).

The level of telomerase activity is associated with the proliferative capacity of cells (Zimmermann et al., 2004) and in vitro mitogenic stimulation upregulates telomerase activity in human lymphocytes despite the very low telomerase activity of unstimulated lymphocytes (Hiyama et al., 1995). The telomerase activity of the subconfluent progeny is intermediate between that of the P6 and P7 CECs and much lower than the spheres from which they are derived. These results suggest that the progeny possesses a higher-proliferative potential than the passaged P7 CECs.

With respect to the clinical application of CEC sheet transplantation in place of full-thickness corneal grafts, the feasibility of harvesting cultured CECs has been reported (Engelmann and Friedl, 1989; Engelmann et al., 1999; Aboalchamat et al., 1999; Bohnke et al., 1999; Chen et al., 2001; Mimura et al., 2004a,b; Ishino et al., 2004). Cultured CECs are an attractive source for regenerative medicine because there are not enough donor corneas anywhere in the world, while cultured CECs could create a number of corneal sheets for transplantation. Passaging of cultured CECs, however, leads to senescence, and the cultured cells become larger and more senescent with older donor age and increasing passage number (Miyata et al., 2001; Zhu and Joyce, 2004; Joyce and Zhu, 2004). The sphere-forming assay could be a useful tool to maximize the number of CECs from a donor cornea and it may even become a standard technique to obtain cells for regenerative medicine.

9. Future directions and summary

DSAEK is safer and dramatically improves vision compared with conventional PKP. This review has highlighted some of the recent experimental innovations in DSAEK surgery using cultured HCEC sheets to overcome the donor cornea shortage. The techniques presented here are good candidates for development to treat CEC dysfunction, but several modifications will be needed before DSAEK can be performed clinically using cultured HCECs. First, the density and quality of HCECs on the DSAEK grafts must be similar to that in vivo, so the cell density of HCEC solutions and the methods of seeding and attachment to the carrier must be improved further. Second, although HCECs from primary culture or early passages are likely to be of better quality, mass culture of HCECs with long-term proliferative capacity should be established to overcome the donor cornea shortage. Additionally, like other methods of tissue engineering for organ regeneration, transplantation of cultured HCECs may raise ethical problems that could interfere with clinical use.

Autologous CEC transplantation is undoubtedly an ideal strategy to completely avoid the risk of rejection. Because CECs from the peripheral cornea contain more precursors than CECs from the central cornea in rabbits (Mimura et al., 2005b) and humans (Yamagami et al., 2007), culture of peripheral cells obtained by resecting a small piece of Descemet's membrane may eventually allow transplantation of HCEC sheets for unilateral bullous keratopathy. Cultured HCECs may eventually become a powerful tool for cell or tissue regeneration and transplantation.

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